

Clinical, laboratorial and immunological aspects of severe malaria in children from Guinea-Bissau

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ABSTRACT

Malaria is a parasitic disease of which *Plasmodium falciparum* causes the most severe form of the disease. The immune response against *Plasmodium* spp. is complex and remains unclear. The present report aimed to better understand the humoral immune response in severe malaria and analyse new immunodominant antigen candidates as possible serological marker in severe malaria in children.

This study included children aged 0–16 years from Guinea-Bissau with clinical signs of severe malaria. Serological and immunochemical characterisation of different anti-*P. falciparum* antibodies were made by ELISA and immunoblotting using a crude protein extract of *P. falciparum*.

Sera from 12 children with severe malaria were analysed. Nine samples were positive for total anti-*P. falciparum* antibodies, seven for IgM and eight for total IgG anti-*P. falciparum*. There was a predominance of IgG1 response, suggesting a cytophilic action in severe malaria and a major role of IgG1 over other immunoglobulins. The antigenic profile of *P. falciparum* showed a consistent immunoblotting pattern of approximately 180 kDa, 100 kDa and around 50–40 kDa.

The serological reactivity found in protein bands makes them as immunodominant antigens and promising candidates for serological markers in the context of severe malaria.

1. Introduction

Plasmodium falciparum causes the most serious form of the disease. Infections with this parasite can be fatal in the absence of prompt recognition of the disease, appropriate treatment and patient management (Anon., 2012). The World Health Organization (WHO) have established a set of clinical and laboratory criteria that defines severe malaria (Anon., 2014).

Immunity seems to require humoral and cellular immune responses, as the passive transfer of purified immunoglobulin (Ig) G from hyper-immune sera to infected subjects has demonstrated that IgG mediates anti-parasitic activity and overall protection against malaria (Cohen et al., 1961). Also, a study of individuals from Ivory Coast and Burkina Faso infected with *P. falciparum* demonstrated that a predominance of

IgG1 and IgG3 cytophilic antibodies was associated with protection against the disease, whereas a predominance of IgG2 and IgG4 non-cytophilic antibodies were related to an increased susceptibility to malaria infection (Bouharoun-Tayoun and Druilhe, 1992). However, the question about which cytophilic antibody is produced first or which one is more relevant remains unclear (Pinto et al., 2001).

On the other hand, IgM has been known to neutralize antigens very effectively, acting as part of an early immune response. Several studies suggest that IgM has a protective role against malaria infection (Boudin et al., 1993). For instance, IgM antibodies and mononuclear cells act synergistically to inhibit the growth of *P. falciparum* in vitro more effectively than IgG (Brown et al., 1986). However, further evidence indicates that the binding of IgM antibodies to the surface of infected erythrocytes via the Fc receptor has a positive correlation with the

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formation of rosettes (Rowe et al., 2002; Ghumra et al., 2008), suggesting a possible role of IgM in the severity of falciparum malaria.

The role of IgE antibodies in protection and/or pathogenesis is not well established. Studies conducted in some islands of Burkina Faso and in the frontiers of Thailand-Myanmar and Thailand – Cambodia indicated that IgE may be related with the severity of malaria, as they showed high levels of IgE in subjects with severe malaria (Calissano et al., 2003). On the other hand, another study from a holoendemic area of Tanzania, suggested that higher levels of malaria-specific IgE in children were associated with a statistically significantly reduced risk of acute malaria (Bereczky et al., 2004).

The wide gap in understanding the biology of *P. falciparum* infection makes difficult to identify the most effective vaccine candidate from among the many of potential antigens of *P. falciparum*. The malaria vaccines that present themselves as current candidates are directed towards stages of the parasite's life cycle in humans and mosquitoes, but until now, relatively few proteins have been studied towards developing a potential vaccine (Crompton et al., 2010). Nevertheless, a yet large number of antigens have presented a major challenge towards the identification of the protective responses and their targets, and it may be that efficient immunity is mediated by responses to multiple antigens.

Despite the global importance of *P. falciparum*, much work is yet to be done regarding antigens characterisation. Therefore, the present study aims to identify and characterise immunochemically the *P. falciparum* antigens involved in the induction and maintenance of humoral immunity, in children with clinical signs of severe malaria caused by *P. falciparum*.

2. Material and methods

2.1. Description of the studied population

This study included 12 children from 0 to 16 years old, with signs of severe malaria or malaria requiring hospitalization due to oral intolerance. All the children included were clinically observed in the paediatric consultations of the Hospital São José de Bor, in the village of Bor, Bissau (Guinea-Bissau). The severe malaria diagnosis was assessed through clinical evaluation and children fulfilling the following inclusion criteria were enrolled: i) clinical diagnosis of severe malaria, according to the clinical criteria established by WHO (2); ii) positive microscopic examination. Children presenting the following medical conditions were excluded: i) diarrhoea and severe dehydration; ii) severe malnutrition; iii) pneumonia; iv) meningitis; v) encephalitis; vi) tuberculosis; vi) diagnosed HIV infection.

An informed consent was applied to every participant and signed by the parent or legal guardian. The participation of patients in the study was voluntary. The study protocol was approved by the ethical committee (protocol number 3–2012-TM) of Instituto de Higiene e Medicina Tropical, Lisbon, Portugal.

2.2. Samples collection

Blood samples were collected by venepuncture into heparin tubes. Approximately 100 µl of total blood was absorbed in Whatman™ filter paper (GE Healthcare, UK) and let dry at room temperature (RT). The remaining sample was stored at –20 °C until needed.

2.3. Laboratorial confirmation of *Plasmodium falciparum* infection

In order to confirm the positive infection of malaria, rapid diagnostic test – RDT (Rapid Malaria pf/pan Antigen Test, Boson Biotech, China), microscopic examinations and Polymerase Chain Reaction (PCR) were performed. The RDT applied uses *P. falciparum* histidine-rich protein 2 (HRP-2) and lactate dehydrogenase (LDH) and was performed according to the supplier's instructions.

Microscopic examination consisted on the visualization of thick blood smears, fixed with 10% methanol (v/v) for 1 min, stained with 1% Giemsa's solution (Merck, Germany) in Phosphate Buffered Saline (PBS) (v/v) for 10 min, washed with water and allowed to dry at RT.

Genomic DNA of *P. falciparum* was extracted using QIAamp DNA Mini Kit (Quiagen, USA), according to manufacturer's instructions. DNA was afterwards amplified by nested-PCR, using primers designed by Snounou et al. (1993), recognising the small subunit (18S) of ribosomal RNA gene, and synthesised by Thermo Scientific (Germany). For PCR reactions the following reagents' concentration was used: a) 10 x NH₄ buffer, 50 mM MgCl₂, 100 mM mixed dNTP, 100 pmol/µl of sense and antisense primers; 5U/µl Taq polymerase, 3 µl DNA and water up to 50 µl. Amplification conditions consisted of a) initial denaturation at 94 °C for 2 min; b) denaturation at 94 °C for 0.30 min; c) annealing at 55 °C for 1.30 min; d) extension at 72 °C for 2 min; repeat step b) to d) for 40 times; e) final extension at 72 °C for 10 min. A negative control (no template added) and a positive control (sample with infected blood) were used. The amplified products were observed in 2% agarosis gel (w/v).

2.4. *Plasmodium falciparum* parasites culture and total protein extraction

Cultures of Dd2 *P. falciparum* clones were developed in complete RPMI 1640 culture medium with 5% haematocrit, 37 °C and an atmosphere with 5% of CO₂ as described in the work of Costa et al. (2013). To extract *P. falciparum* proteins, parasite cultures were transferred to 15 ml tubes, centrifuged at 2500g for 2 min at RT and the supernatant was discarded. Cells were suspended in lysis buffer (5 mM sodium phosphate, pH 8 + protease inhibitor [Complete Ultra Tablets, Roche, Switzerland]), five times the pellet volume. The tubes were placed on ice for 15 min and then centrifuged at 18000g, at 4 °C for 20 min. The supernatant was discarded. The pellet was washed three times with ice cold PBS. Every wash cycle comprised the addition of PBS and a centrifugation at 18000g for 10 min at 4 °C. The supernatant was discarded at every wash cycle. The parasite lysis was accomplished with four times the pellet volume of lysis buffer (0.1% Triton X-100 in PBS + protease inhibitor [Complete ULTRA Tablets, Roche, Switzerland]). The tubes were placed on ice for 30 min with gently shaking, and homogenised by vortex every 10 min. After, the tubes were placed at –70 °C for 5 min and then at 37 °C for 5 min. This process was repeated two more times. After this cycle, the samples were centrifuged at 18000g for 10 min at 4 °C. The supernatant was recovered and stored at –20 °C. The quantification of total protein of *P. falciparum* was performed with Bicinchoninic Acid Protein kit (Sigma, USA), according to the manufacturer's protocol.

2.5. Serological measurement of anti-*Plasmodium falciparum* antibodies

Total antibodies (IgG + A + M), IgM, total IgG and its subclasses and IgE were detected by enzyme-linked immunosorbent assay (ELISA) using crude protein extract of *P. falciparum* to coat 96-well microplates (Thermo Fisher Scientific, USA). The assay was developed according to Costa's protocol (Costa et al., 2013), with few changes: wells were coated with 200 ng/well of crude protein extract of *P. falciparum* in bicarbonate buffer (0.1 M, pH 8.5). The primary antibody was diluted 1/100, except when measuring total IgG, which was diluted 1/200. The secondary antibody consisted of several different antibodies (used in separated assays), with different dilutions, different substrates and stop solutions applied.

All samples were tested in duplicate and positive and negative controls were used (adults with acute malaria and children without stays in endemic areas of malaria, respectively). For the serological measurement of IgE and IgG subclasses, neither positive nor negative controls were used, for lack of truly representative control samples.

The cut-off was established as the mean of the negative controls plus a standard-deviation (SD), obtained for the different antibody analysed.

The results were expressed in OD/cut-off to be compared between assays. Samples with OD/cut-off ratio above 1.1 were considered as positive and under 0.9 were considered to be negative for malaria antibodies. Values in between 0.9 and 1.1 were repeated and, when the result remained, were considered undetermined.

2.6. Characterisation of *Plasmodium falciparum* proteins by immunoblotting

Western Blot was performed as described in Calisto (2015), using 23 ng/well of crude protein extract of *P. falciparum*. The primary antibody was diluted 1/100 (v/v) in antibody buffer (1% blocking buffer in wash buffer). The determination of the protein's molecular weight was estimated by comparison with a 10–190 kDa commercial molecular weight marker (HyperPAGE Prestained Protein Marker, Bioline, UK). As negative controls, sera from subjects who have never been in malaria-endemic areas were used. As positive controls, sera from patients infected with *P. falciparum* were used.

2.7. Ethics approval and consent to participate

This study protocol was approved by the ethical committee of Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal. Protocol number 3–2012-TM.

3. Results

In this study 12 children were included with a mean age of 6.75 years (SD = 1.194; min = 2, max = 14). The majority of the children was female, representing 58% of patients (n = 7). The clinical and laboratory results of the examination at admission are summarised in Tables S1 and S2.

To confirm the infection by *P. falciparum*, RDT, microscopy and PCR were performed. The results are presented in Table 1. Eleven samples were positive for *P. falciparum* in RDT and microscopy, but all the 12 samples were positive when tested by PCR. Children denominated of P3 and P5 showed a possible co-infection with *P. falciparum* and *P. malariae* according to the results obtained by RDT. The sample P11 was weakly positive for *P. malariae* by RDT and it was the only sample that was negative in microscopy. PCR was performed for the amplification of *P. falciparum* DNA and all samples were positive.

The levels of total antibodies (IgG + IgA + IgM), IgM, IgG and its subclasses and IgE were determined in the twelve infected children with severe malaria. The results are presented in Fig. 1A. From the twelve samples, nine (75%) were positive for total anti-*P. falciparum* antibodies, seven (58.3%) were positive for IgM, with one sample considered as undetermined. IgG anti-*P. falciparum* showed eight (66.7%)

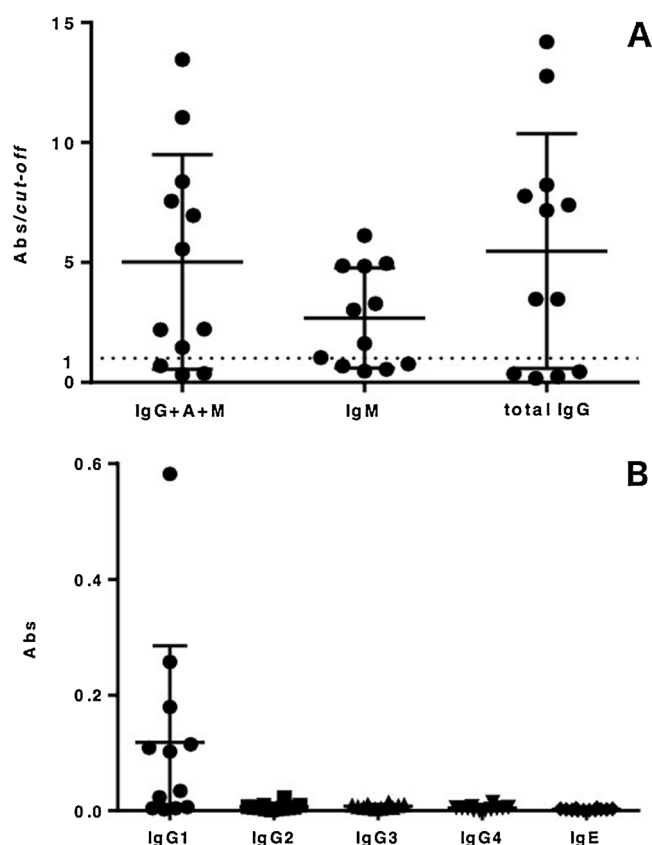


Fig. 1. Serological levels of anti-*Plasmodium falciparum* antibodies, namely total antibodies (IgG, IgA, IgM), total IgG, total IgM, IgG isotypes (IgG1, IgG2, IgG3, IgG4), and IgE performed by indirect ELISA.

positive samples (Table 2, Fig. 1A). The levels of total IgG were almost always higher than IgM, except for sample P13. The levels of IgG subclasses were assessed in the 12 *Plasmodium*-infected children, in order to characterise the nature of the humoral response in the population studied. A predominance of the cytophilic IgG1 response was observed, with no serologic reactivity of any other subclasses (Error! Reference source not found.1B). None of the samples tested was positive for IgE antibody (Table 2).

The *P. falciparum* antigens responsible for the serological reactions by ELISA assays were detected by immunoblotting. The antigenic profile of the population studied is presented in Fig. 2. The analysis of the antigens profile of the samples from children with severe malaria showed a consistent reactive pattern around the molecular weights of

Table 1
Laboratory confirmation of malaria infection and results of serologic assays.

Samples	Microscopy	RDT		PCR (<i>P. falciparum</i>)	Total Abs (abs/cut-off)	Total IgG (abs/cut-off)	IgM (abs/cut-off)	IgE (abs/cut-off)
		<i>P. malariae</i>	<i>P. falciparum</i>					
P1	+	–	+	+	11.05	12.77	6.12	0.004
P2	+	–	+	+	2.22	3.47	0.68	0.001
P3	+	+	+	+	7.56	8.23	3.28	0.006
P5	+	+	+	+	0.69	0.25	0.76	0.001
P6	+	–	+	+	2.19	3.47	1.03	0.002
P7	+	–	+	+	0.37	0.36	0.54	0.001
P8	+	–	+	+	13.46	14.20	4.86	0.005
P9	+	–	+	+	8.37	7.77	4.84	0.003
P10	+	–	+	+	5.56	7.39	3.02	0.001
P11	–	+/-*	–	+	0.32	0.17	0.47	0.001
P12	+	–	+	+	6.96	7.17	4.96	0.004
P13	+	–	+	+	1.46	0.44	1.62	0.002

*Weakly positive.

Table 2

Potential *P. falciparum* antigens responsible for the positivity for total IgG antibodies. Only the most reactive protein bands in immunoblotting were considered.

Protein Mass (kDa)	Representatively in the population studied (%)	Potential antigen candidates	References
182	67	MSP-1 precursor 175 K antigen	Freeman and Holder (1983) Camus and Hadley (1985)
97	83	96tR antigen	Jouin et al. (1987)
50	58	51 kDa glycosylated antigen	Epping et al. (1988)
42	59	MSP-1 (MSP1) ₄₂	Blackmann et al. (1991)

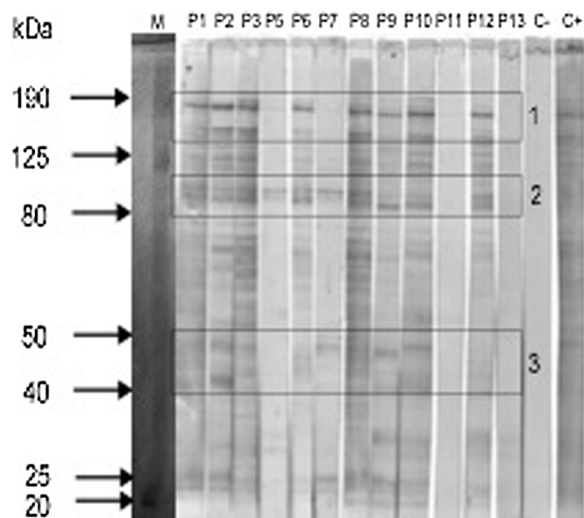


Fig. 2. Protein pattern of the reactivity detected by immunoblotting using 23 µg/well of *Plasmodium falciparum* crude extract. M: molecular weight marker HyperPage (Bioline, UK). C-: negative control; C+: positive control.

190 kDa and 100 kDa (Error! Reference source not found.2, region 1 and 2). Notwithstanding, another (less relevant) pattern seems to emerge in the molecular weight of approximately 50 kDa and 40 kDa (Fig. 2, region 3).

Comparing the protein molecular weight marker with the most reactive proteins obtained, it is possible to estimate potential *P. falciparum* antigens that were responsible for the IgG anti-*P. falciparum* antibodies detected through ELISA. That analysis is presented in Table 2.

4. Discussion

The present study aimed to characterise the mechanism of an early humoral immune response of severe malaria, by serologic and antigenic reactivity during the first/former *Plasmodium* infections. The levels of IgG measured indicated its importance for parasite control.

A group of 12 children from Bissau, Guinea-Bissau, with severe malaria were included. Laboratorial diagnosis confirmed the infection with *P. falciparum*. Sample P11 may seem contradictory but several explanations may exist: (i) sensitivity/specificity of the methods; (ii) low parasite load; (iii) poor quality of the sample. Also, the polymorphism in the *hrp-2* gene of *P. falciparum* may affect the sensitivity of the HRP-2 based RDT (Baker et al., 2010; Baker et al., 2005; Kumar et al., 2012). For LDH, there is no polymorphism described, but the detection of this antigen may fail due to the low sensitivity of the test in cases of low parasitemia (Jang et al., 2013). Due to the higher sensitivity and specificity of PCR regarding the other two methods, sample P11 was still considered for further analyses (Mahende et al., 2016).

The results obtained by ELISA showed that the majority of the children included have started developing the humoral response already. The three negative samples may correspond to recent infection, with antibodies at undetectable levels in in vitro assays or with no time

to orchestrate the immune response.

Comparing the levels of those IgG and IgM antibodies, IgG antibody was presented in higher levels than IgM, suggesting that IgG is important for parasite control, as indicated by Richards et al. (2010). Only sample P13 had a higher level of IgM than IgG, indicating that child did not develop a specific humoral response until blood collection.

In this present work, the mean age of the children with anti-malaria antibodies was 9.4 years, and without anti-malaria antibodies was 4.5 years, allowing to speculate that older children can mount stronger humoral responses, perhaps due to more exposures to the parasite in the past. Analogous results were described in the study of Richards et al. (2010).

The nature of the humoral response in severe *P. falciparum* malaria seems to be characterised by a predominance of the cytophilic IgG1. Pinto et al. also reported a dominance of cytophilic antibody rather than non-cytophilic subclasses, when evaluating the response of IgG subclasses anti-*P. vivax* in 34 children from 0 to 15 years old. In particular, patients showed a predominant IgG1 response, where 28 of these children had never had any previous history of malaria and 6 had one or two episodes of malaria (Pinto et al., 2001).

Since cytophilic antibodies are known to promote phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) processes (Bouharoun-Tayoun and Druilhe, 1992; Ferrante et al., 1990), the results of this present report suggest that these antibodies are involved in opsonisation of infected red cells, as indicated elsewhere (Groux and Gysin, 1990; Tebo et al., 2002). Furthermore, there is also evidence that these subclasses antibodies act synergistically with monocytes through antibody-dependent cellular inhibition (ADCI) processes, strongly inhibiting parasite growth (Theisen et al., 1998; Singh et al., 2005; Shi et al., 1999; Tebo et al., 2001). There was no reactivity with the others IgG subclasses studied, suggesting that they are not implicated in protective responses in severe malaria.

Regarding IgE anti-*P. falciparum* antibodies, all the twelve samples were negative and unfortunately, no suggestions can be made concerning the controversy between the protective role (Bereczky et al., 2004; Duarte et al., 2007) or the contribution to the severity of malaria (Calissano et al., 2003; Perlmann et al., 1994). As the use of biotine-streptavidine complex increases the sensitivity of the technique, more analyses could be performed to discard the possibility of IgE levels are being underestimated. The confirmation of the serological assays with a larger sample size and with populations in different stages of the disease would be useful to better understand the role of IgE in malaria.

The results obtained in ELISA assays are concordant with the reactivity presented in immunoblotting. Almost all samples showed serologic reactivity, with the exception of P5, P7 and P11 that, similarly to ELISA results, showed a very modest reaction in Western Blot or no reactivity at all. These observations suggest that IgG is required for protection against the parasite in severe cases of the disease. Similar results were found in studies of immunoglobulin transfer of immune donors to patients with acute *P. falciparum* malaria (Cohen et al., 1961).

The analysis of the antigens profile of the samples from children with severe malaria showed a consistent reactive pattern of approximately 100 kDa and 180 kDa. These results are similar to those obtained in a study of 321 uninfected adults that showed IgG antibodies many years after their last stay in endemic areas (Calisto, 2015). Notwithstanding, another pattern seems to emerge between the molecular

weights of 50–40 kDa, having similarities with a study of Costa and colleagues with 227 adults with imported uncomplicated malaria (Costa et al., 2013). It is interesting to notice that the antigenic reactivity founded in the present report shares two different patterns, consistent with two different populations.

The antigenic reactivity in P5 and P7 samples by immunoblotting while negative in ELISA is probably the reflect of the higher sensitivity and specificity of the immunoblotting over ELISA (Palaeya et al., 2013; Cheong et al., 2013). However, we know that the sample population in this study is reduced and therefore this information should be explored in future studies. These results, though only suggestions towards the identification of proteins that reacted with IgG antibodies specific for *P. falciparum*, can prove to be relevant in the development of new protein targets as vaccine candidates.

Further in silico analysis of those antigens should be performed in order to fully characterised them and use them in new serological markers and/or vaccine designs. The knowledge of serological markers, specifically in severe malaria, is important to understand the evolution of the disease and avoid severity. The characterisation of the immune response and the antigen candidates achieved with this work can be useful to develop diagnostic tests in order to identify the severe stage of malaria as well as new and more promising vaccine candidates.

On the other hand, we also understand the importance of inflammation, lymphocytes migration and cytokines involved in the pathogenesis of severe malaria, however we intend to evaluate these components in future studies.

Authors' contributions

Conceived and designed the experiments: AC, LV, MSS.
 Performed the experiments: JD, AC, DPC, FN.
 Analysed the data: JD, AC, DPC, LV, MSS.
 Wrote the paper: JD, AC, DPC, MSS.
 All authors read and approved the final version of the manuscript.

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